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The pharmaceutical Impacts of honeybee venom against thioacetamide-induced hepatic fibrosis in rats

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Abstract

Hepatic cirrhosis is an acute disease accompanying fibrosis, liver cell damage, and liver dysfunction. The current study, the prospective therapeutic effects of honey bee venom (BV) on liver fibrosis were examined in rats administered thioacetamide. Hepatic histology, Masson's trichrome, anti-oxidants (total glutathione and superoxide dismutase), apoptosis and biochemical hepatic functions assays were estimated. We found that BV treatment up-regulated the albumin protein, anti-oxidant enzymes (GSH and SOD) and down-regulated aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, collagen formation and apoptotic rate, which were altered by TAA inducer. Together, these responses increased liver cells sensitivity to TAA-induced hepatotoxicity and forced the damaged cells to undergo apoptosis. Enhancing the tendency of damaged liver cells to undergo apoptosis could be a protective mechanism whereby BV suppresses inflammatory responses and liver fibrosis. The study suggested that honeybee venom prevented TAA-induced liver fibrosis by inhibiting liver inflammation; decreased the high rate of lethality; alleviated hepatic histological injury; attenuated hepatic inflammatory responses; and inhibited hepatic cells apoptosis. These results suggest that honeybee venom could be an effective agent for preventing liver fibrosis.

Keywords: Hepatic fibrosis, Honey bee venom, Thioactamide, Antioxidants

1. Introduction

Hepatic fibrosis is a chronic disease commonly occurs before the cirrhotic and continuing harm to the liver and Kupffer cells. It is a process of cirrhotic liver production, combined with most chronic diseases of liver (Ueberham et al., 2003). Advanced liver fibrosis results in liver failure, cirrhosis, and portal hypertension and often requires liver transplantation. Oxidative stress plays a critical role in activation of hepatic stellate cells (HSCs), which cause the accumulation of extra-cellular matrix (ECM) proteins, in particular collagen, in liver tissue (Friedman, 2003, Fowell and Iredale, 2006). All clinical cases and experimental chronic requirements of liver disease are due to free radicals of oxygen. Lipid peroxidation aldehydes are involved in the development of liver cirrhosis (Mahmood et al., 2004).

Halo-alkanes such as chloroform, iodoform and carbon tetrachloride have been widely used as chemical inducers of liver fibrosis. Similar to carbon tetrachloride, thioacetamide (TAA) also induces fibrosis in liver. The cell toxicity of each compound is due to not to the compound itself but its metabolites (Chilakapati et al., 2007, Weber et al., 2003). Thioacetamide (TAA) using in liver fibrosis in animals has many advantages, including specific progression of human hepatic fibrosis, hepatotoxicity and hepatic damage by chronic liver injury.

However, it remains a problem to prevent cirrhosis or control its progression. Huge efforts have been created to find effective and safe drugs. Various studies of either animal models or human patients have shown that recovery and remodeling of hepatic fibrosis is possible (Lou et al., 2010). Recently, increasing attention has been directed to the curative properties of products of honeybee (bee venom, pollen, propolis, royal jelly, honey and beeswax) in the health treatment conditions (Apitherapy).

Bee venom (BV) is synthesized by the venom glands of the stinger of a bee workers. It is stored in the reservoir of venom, and injected via the stinger into invaders. BV production increases during the first two weeks of the adult worker's life and reaches a maximum secretion during colony defence, and diminishes as the bee achieves older (Ali, 2012). BV is a complex mixture of several biologically active compounds, e.g. peptides, enzymes, and low components' molecular. BV contains a variety of at least eighteen active components with some pharmaceutical properties including (in dry weight): phospholipase A2 (12-12%), melittin (40-50%), apamin (2-3%), hyaluronidase (1-2%), mast cell-degranulating peptide (2-3%), adolapin (0.5-1.0%). Bee venom also contains small amounts of amino acids (1%), phospholipids (1-3%), volatiles (4-8%), sugars (2-4%), minerals

(3-4%)and biogenic amines (0.8-3.5%) (Park et al., 2004, Wang, 2009, Bogdanov et al., 2012,).

Bee venom therapy has been used since ancient times in traditional medicine by direct bee stinging in trigger or acupuncture points to treat inflammation, e.g. back pain, arthritis, rheumatism, and skin diseases. BV has anti-viral, anti-bacterial, and anti-inflammatory activities (Park et al., 2010 a,b, Park et al., 2011). Melittin could be an effective agent for preventing liver fibrosis (Park et al., 2011) and a novel targeted therapy for some types of cancer such as prostate and breast cancer (Oršolić, 2012). Bee venom has substantial therapeutic potential for the treatment of fibrotic diseases (Kim et al., 2010). It is considered an alternative therapy to treat multiple sclerosis (Hauser et al., 2001), Chronic fatigue syndrome and Lyme disease. BV demonstrated antitumor effect and has inhibitory effect on serum C-reactive protein levels (Karimzadeh et al., 2012).

Recent studies have indicated that administration of honey bee venom can significantly impart an anti-arthritic response mediated by inhibition of inflammation mediators similar to non-steroidal anti-inflammatory drugs (Lee et al., 2004, Kim et al., 2013).

As Apitherapy has been frequently investigated for its hepatoprotective and anti-fibrotic effects in both humans and animal models, the aim of the present work was to examine whether bee venom has hepatotherapeutic properties against a rat model of hepatic fibrosis induced by thioacetamide administration.

2. Materials and Methods

2.1. Animals

Experiments were performed on 80 male Sprague-Dawley rats (obtained from the Laboratory Animal House of Faculty of Science, Damanhour University, Damanhour, Egypt) weighing 100–150 g at the start of the experiment. Animals were kept in a cycle 12:12 light/dark in a temperature controlled room (25±2°C). Food and water were available *ad libitum*. All of the methods used in the present study were approved by the Animal Care and Use Committee at Damanhour University and conforming to National Institutes of Health (NIH) guidelines.

2.2. Thioacetamide and route of administration

Thioacetamide (TAA) was purchased from Sigma Company (USA). It was administered at a single dose of 150 mg/kg body weight intraperitoneally (i.p.) according to Mangipudy et al., (1996). The rats received bee venom (1mg/kg) daily through an orogastric tube for 8 weeks.

2.3. Bee venom

A set of Carniolan hybrid honeybee, *Apis mellifera* L., colonies situated in the apiary of the Faculty of Agriculture, South Valley University, Qena, Egypt were used for bee venom collection during spring season. Honeybee venom was obtained based on electric shock method using the device modified by Mohanny (2005).

2.4. Animal grouping

Rats were randomly divided into four groups (n = 20/group): 1) normal (control), 2) bee venom group (1mg/kg), 3) thioacetamide (TAA) (200 mg/kg), and 4) TAA (200 mg/kg) + bee venom (1mg/kg). On the final day of experiment animals were fasted for 12 hrs, and weighed, for sacrifice preparation. The animals were diethyl ether anesthetized. hepatic tissues were rinsed in an isotonic saline, quickly dissected, dried by pieces of filter paper, and stored at -20°C in plastic vials for examination. Frozen hepatic tissue samples were subjected for estimation of antioxidant reduced superoxide dismutase (SOD) and glutathione estimation contents. Other samples of the hepatic tissue were stored in neutral buffer formalin for histological studies.

2.5. Biochemical assays for liver functions

The blood was collected from abdominal aorta using syringes for biochemical investigations. Blood serum was stored at -80°C until measurement of alanine aspartate aminotransferase (AST), aminotransferase (ALT), albumin and bilirubin. Automatic analysis were used as the biochemical markers for the early acute hepatic damage (Jesse et al., 2009).

2.5. Total glutathione (GSH) and superoxide dismutase (SOD) estimation

The homogenized liver tissue GSH level was assayed according to Beutler et al. (1963). This method utilized meta-phosphoric acid for protein precipitation and 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) for color development measured at 412 nm. The homogenized liver tissue Superoxide dismutase (SOD) was assayed according to Misra and Fridovich (1972) using specified kits (Randox-Ransod, Crumlin, Co., UK). The change in extinction coefficient was measured at 480 nm for SOD.

2.6. Histological and Masson's trichrome staining

All liver tissue specimens were fixed in 10% formalin for at least 24 hours at room temperature (25±2°C). After fixation, the tissues sections were dehydrated in ascending ethanol series, cleared in xylene, and then embedded in paraffin. Thin sections (3µm) were mounted on glass slides, deparaffinised, rehydrated with descending ethanol series and distilled water, and stained with hematoxylin and eosin for histopathological examination. Cross sections taken from the hepatic tissues blocks were stained with Masson's trichrome for collagen estimation.

2.7. TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique, which investigates fragmentation of DNA in the nucleus during apoptotic cell death process, was employed using an apoptosis detection kit (Calbiochem, USA). Hepatic tissues sections of 4µm thickness were deparaffinized in xylene and rehydrated through a graded ethanol series. They were then incubated with 20 mg/ml proteinase K for 20 min and rinsed in Tris-buffered saline (TBS). Endogenous peroxidase activity was inhibited by incubation with 3% H_2O_2. Hepatic sections were then incubated with equilibration buffer for 10–30 min and then in terminal deoxynucleotidyl transferase (TdT), in a humidified atmosphere at 37°C, for 90 min. They were subsequently put into pre-warmed working strength stop/wash buffer at room temperature (25±2°C) for 10 min and incubated with blocking buffer for 30 min. Between each step and the subsequent one sections were separated by washes in TBS. Labeling antibody was showed using diaminobenzidine (DAB), counter staining was completed using methyl green, and then sections were dehydrated, cleared and mounted. The number of hepatocytes TUNEL-positive, including hepatocytes and non-parenchymal cells, in each specimen was recorded.

2.8. Statistical analysis

The obtained data were analysed using one-way ANOVA and expressed as means ±SD. Student's t-test was used for comparison between groups. $p<0.05$ was considered to be statistically significant. The SPSS, 12.0, software was applied.

3. Results

3.1. Biochemical analysis

Data given in Table (1) show that the hepatic cytoplasmic. hepatic enzyme activities (ALT & AST) were increased significantly ($p<0.05$), while albumin was decreased significantly in the TAA and TAA+BV groups compared to the control and bee venom groups. Bee venom alone could approximate the activities of these enzymes in the *control* group.

Table (1): Data of biochemical analysis (mean ± SD) in different animal groups

Animal groups	ALT (U/l)	AST (U/l)	Albumin (g/dL)	Bilirubin (mg/dL)
Control	40.44±0.98	39.32±2.01	4.18±0.81	0.48±0.02
BV	38.98±0.35	42.24±1.99	4.88±0.95	0.38±0.11
TAA	77.81±13.04*	210.61±25.01*	1.91±0.68*	2.23±1.08*
BV+TAA	45.29±8.19	70.91±9.09*	3.69±0.17	1.02±0.50*

* $P<0.05$ (For significance).

3.2. Anti-oxidant enzymes analysis

In figure (1), the mean hepatic SOD and GSH levels of TAA group were significantly ($p<0.05$) higher than the TAA treated with BV group, while in the control and in the BV only-treated groups, the mean plasma SOD and GSH levels were not significant, respectively. The treated animal group (BV+TAA) restored significantly the depletion in GSH content and SOD activity ($p<0.05$ and $p<0.01$, respectively) compared to the TAA group.

3.3. Histopathological examination

As illustrated in **Figure (2)**, hepatic tissue in the normal control group showed that hepatic cords were ordered in radiating fashion around the hepatic central vein (CV) and the parenchymal plates were divided by non-congested sinusoids. Hepatic fibrosis induced by TAA disrupted this normal lobular architecture. Changes with fibrosis were commonly accompanied by significant hepatic sinusoidal congestion, bile pigment distribution, necrosis, dilated of the central and portal veins and infiltration of lymphocytes. Improvement in hepatocytes occurred in BV treatment compared to those observed in the degenerated hepatocytes, except for some inflammatory cells and mild central vein congestion.

Histopathological changes in fibrosis occurred in TAA-treated rat livers, and their prevention by treatment with BV was observed (**Fig. 3**). The collagen of these fibrotic tissues had a blue color when stained by Masson's trichrome. In control and BV animals, the liver sections showed normal hepatic cells without fibrosis (**Fig. 3A-B**). The livers of rat that were treated with TAA showed extensive accumulation of thick fibrotic tissue, resulting in the formation of nodules of regeneration, fibrotic septa, and noticeable alterations in the central vein compared to the control (**Fig. 3C-E**). The lesions of BV-treated rat were present to a lesser degree (Fig. 3F) than those found in the TAA-treated group. The accumulation of collagen fibers with fibrotic septa was also shown in the severe damaged liver. BV treatment decreased the size of the fibrotic region with less severe progression of liver fibrosis. These results showed that BV contributed to the inhibition of liver fibrosis.

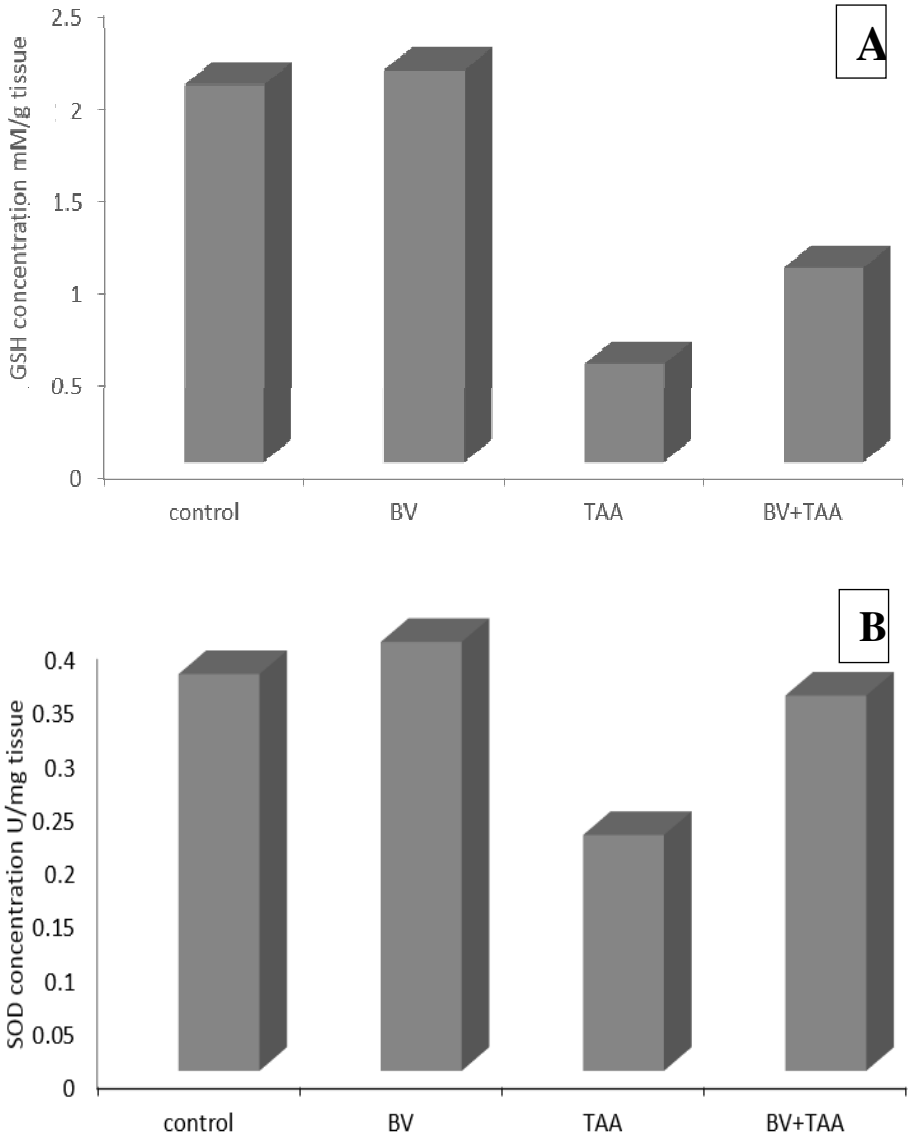

Fig. 1. A) liver tissue GSH (mM/g) levels and (B) liver tissue SOD (U/mg) levels in different experimental animal groups.

3.4. TUNEL findings

Apoptotic index for each group is shown in **(Fig. 4)**. Within the cell cords and the sinusoids, a number of TUNEL positive cells were noticed. The strong TUNEL-positive cells was shown in the TAA group (10.34 ± 1.25, Fig. 4C) compared to the other animals groups. A statistically significant reduction of hepatocyte apoptosis in TAA+BV group (4.98 ± 0.78, Fig. 4D) was shown when compared to the TAA injected group ($p < 0.001$). It was also observed that BV group (1.96 ± 0.30, Fig. 4B) was similar to the control group (1.63 ± 0.56, Fig. 4A) and also the BV group exhibited non-significant alterations in TUNEL positive cells compared to the control group.

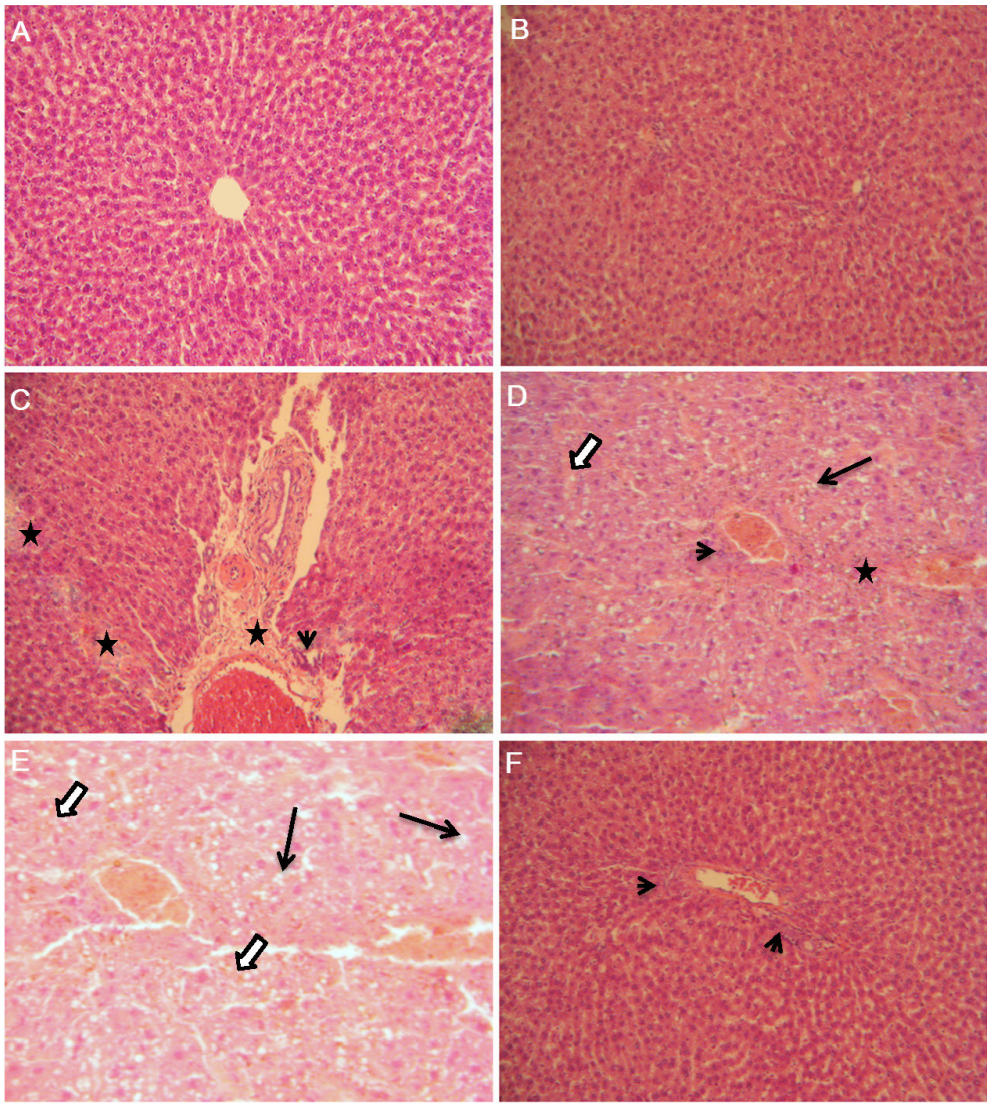

Fig. 2. Rat liver sections stained by hematoxylin and eosin. (A) and (B) showing normal hepatic architecture of control and BV groups. (C-E) showing advanced cirrhosis of *TAA* group with numerous vacuolation (arrows), dense collagenous matrix containing many fibroblasts (stars), bile pigment distribution (white arrows) and inflammation with disturbed hepatocytes (head arrows). (F) showing a reduction in the histological injury as a few hepatic lymphocytes infilteration, thinner fibrous septa, slight dilatation and vacuolation and reduced fibrous tissue proliferation at the portal areas. (Original magnification 200X)

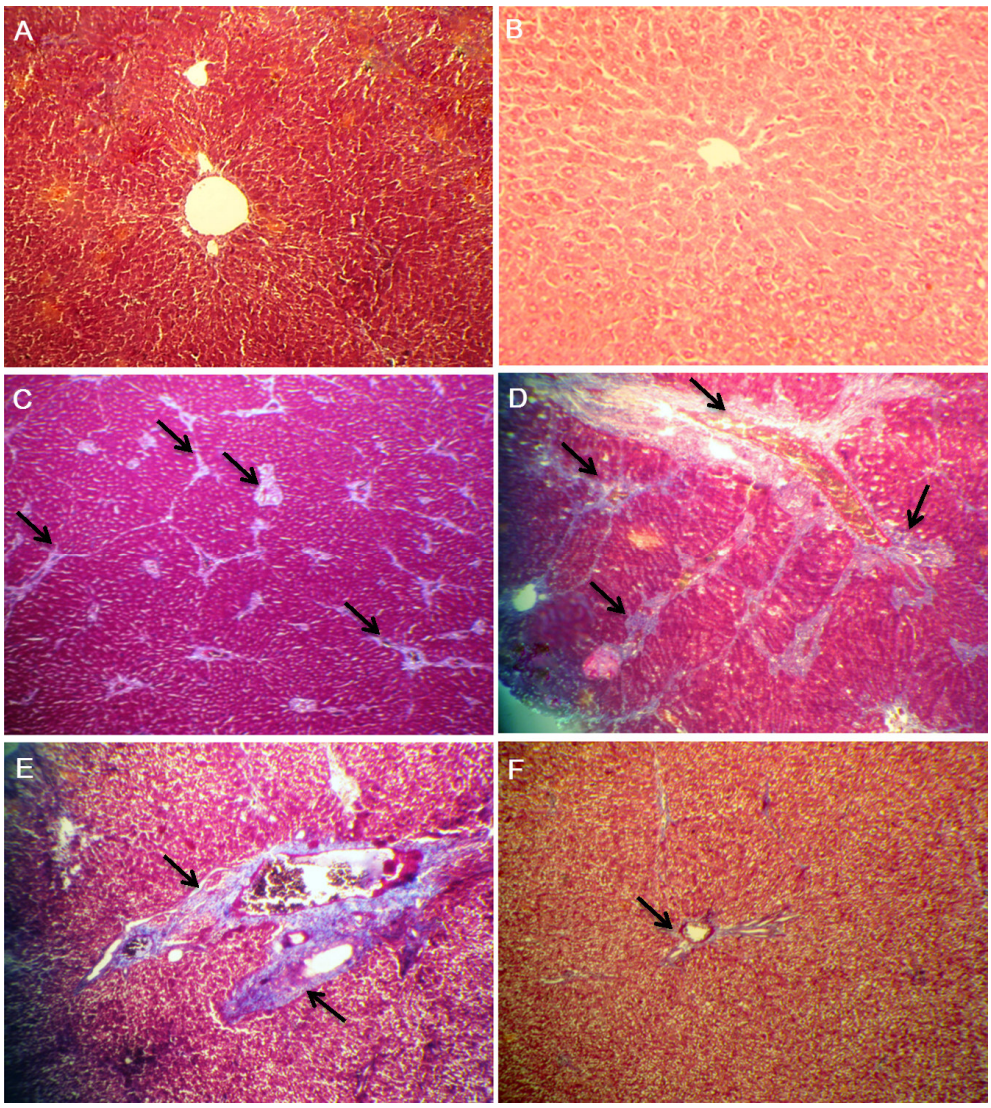

Fig. 3. Rat liver sections stained by Masson's trichrome. (A, B) liver section of control and BV groups, respectively shows no collagen deposition around central vein. (C-E) liver sections of TAA-treated rat show severe deposition of collagen, around portal triad. Arrows point to the collagen fibers. (F) liver section from TAA+BV-treated rat shows significant marked reduction of collagen deposition. (Original magnification X 200)

4. Discussion

Investigation of concentrations of liver enzymes is one of the most sensitive blood tests applied to the diagnosis of liver functions (Kim, 2008). In the current study, the increased serum concentrations of AST, ALT, bilirubin and decreased serum concentrations of albumin protein in TAA-induced hepatic fibrosis rats showed the sign of hepatic cells damaged. Albumin is a rough measure of blood proteins reflects faulty liver to carry out its vital functions and nutritional condition (Lee, 2012). Hepatic enzymes, *i.e.* AST, ALT are normally presented in hepatic cell cytoplasm and are only released into the blood stream when the hepatic cells are damaged, as well as exalted concentrations of the enzymes which reflect hepatic dysfunction (Giannini et al., 2005). After honeybee venom treatment, the lowered serum levels of ALT, AST and bilirubin and the increased serum albumin concentration were observed, and this is in the same line with histological examination. The current results suggest that BV has the possibility of maintaining the integrity of liver cirrhosis resulting from TAA in a rat model. The bilirubin retention in the liver resulted in hepatic cells damage arising from a rapid inflammatory as the reaction including the activation of Kupffer cells and the accumulation of inflammatory cells (Saito and Maher, 2000).

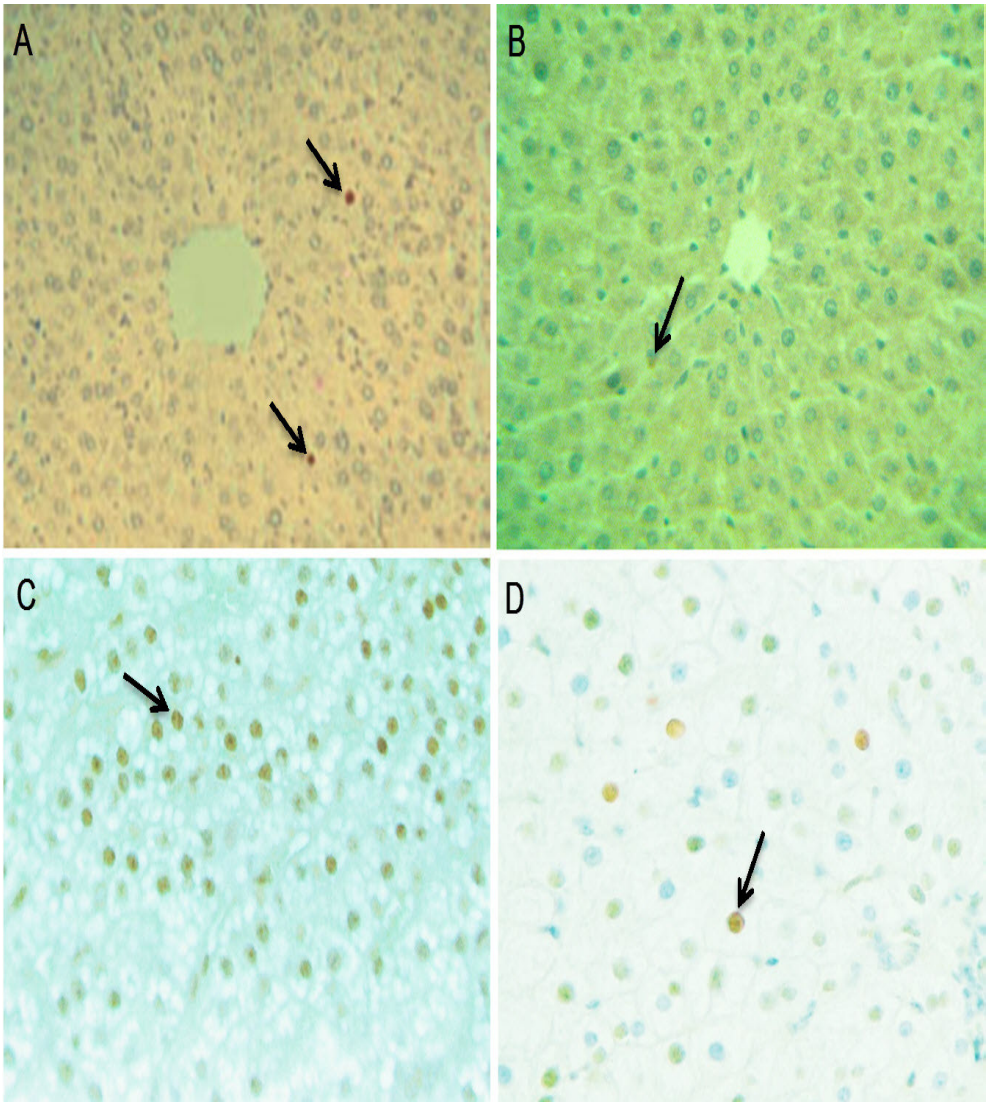

Fig. 4. Labelled nuclei brown (arrows) of apoptotic cells with TUNEL method. **(A & B)** *control* and *BV* groups, respectively, where TUNEL positive cells are shown. **(C)** *TAA* group with numerous TUNEL positive cells. **(D)** *TAA+BV* group with recover sign, where the regression in number of TUNEL positive cells was noticed. Counter staining was methyl green.

Results showed that TAA- induced oxidative stress was evidenced by a significant decrease in GSH and SOD contents. Activities of the anti-oxidant enzymes SOD and GSH in liver samples were decreased by injection of TAA. The same trends have been recorded in literature after induction of cirrhotic liver in animals. BV reduced the oxidative stress possibly by scavenging of free radicals produced by TAA toxicity with subsequent restoration in oxidative stress indicators, where SOD and GSH concentrations in liver homogenates showed relatively similar findings with those reported in other hepatic dysfunction studies (Khan and Ahmed, 2009, Khan et al., 2012).

In the present study, we examined the effects of BV in liver fibrosis following chronic injuries by TAA and explored the modes of actions related with liver fibrosis and inflammation. The histological results basically supported the results of serum enzyme assays. The histopathological investigations of the rat treated with BV and TAA showed normal architecture of the liver having inverted to a large extent, *i.e.* the liver lesions formed by the TAA toxin. Inflammatory lesion is commonly related to liver fibrogenesis during chronic liver disease (Marra, 2002). Where, the induction of oxidative stress is a key feature in the destruction of parenchyma and leads to necrosis, fibrosis and cirrhosis (Poli, 2000). Noticeably, staining by hematoxylin and Masson's

trichrome was a remarkable indicator for hepatic fibrosis after such treatments. The hepatocytes damage is due to the direct subclass of intrinsic hepatotoxicity. TAA is known to exert its action through hepatocyte membrane peroxidation by free radicals, whereas indirect hepatotoxin is suggested to act through its metabolites reacting with intracellular molecules or the cellular membrane to disrupt cellular integrity. Obtained results showed that fibrosis in the portal area was significantly more serious, as observed with hematoxylin & eosin and Masson's trichrome staining, indicating that this region suffered more serious damage than did the peri-central area. The level of exogenous harmful substances in the blood is higher than that in the liver portal zone. This is because the blood stream moves into liver via hepatic portal vein, then from this region to the hepatic central vein. The hepatocytes might have gradually got rid of the toxin as they have a strong function of removal toxins, making the level of toxins decreased from the liver portal area to the central vein. This is the possible explanation for this fibrosis phenomenon which induced by TAA.

Apoptosis is a physiological process that entails the programmed death of a cell and indicates tissue damage. Apoptosis plays a significant role in different liver diseases and associated injuries (Liu et al., 2004). Death of hepatocytes typically follows one of two models, necrosis or apoptosis. To date no literatures about the TUNEL-positive apoptotic cells on TAA-induced liver dysfunction with honeybee venom treatment in rats have been reported. However, in the present study, when hepatic sections were stained TUNEL, there was an increase significant in the number of positive cells in the TAA-treated rats in the hepatocytes. Honeybee venom treatment markedly decreased the reactivity and the number of TUNEL-positive cells in TAA-induced hepatic fibrosis in rats.

In a conclusion, honeybee venom protected against TAA-induced liver fibrosis by suppressing liver inflammation and fibrogenesis. BV decreased the high rate of lethality, alleviated hepatic histological injury, attenuated hepatic inflammatory responses, and inhibited hepatic cells apoptosis. Therefore, the present study proposes that BV may be useful as a potential therapeutic agent for attenuating hepatic fibrosis.

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